Expression of TP and TIE2 genes in normal ovary with corpus luteum and in ovarian cancer: correlation with ultrasound-derived peak systolic velocity

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Transvaginal colour and pulsed Doppler ultrasonography analyses of blood flow velocity have indicated that intra-tumoral peak systolic velocity (PSV) is a good indicator of ovarian malignancy. Therefore, we examined whether there was an association between the expression of angiogenic genes, e.g. thymidine phosphorylase (TP) and TIE2 and the PSV of blood flow in normal and cancerous ovaries. Initially, 40 patients were examined by transvaginal ultrasonography and 23 ovaries were surgically removed; 14 were normal with corpora lutea (CL) and nine showed ovarian cancer. The ovarian tissue was dissected according to areas of high blood velocity and gene expression was examined using the reverse transcriptase–polymerase chain reaction (RT–PCR). No significant differences were found between PSV in the normal ovary with CL and ovarian cancer (P = 0.95). TP gene expression was significantly higher in ovarian cancer than in normal ovary with CL (P = 0.02), while TIE2 gene expression was not significantly different (P = 0.186). There was a significant correlation between TIE2 gene expression and PSV in the normal ovary with CL (r = 0.633, P = 0.015), while TP expression was significantly correlated with the PSV in ovarian cancer (r = 0.757, P = 0.018). These results indicate that there is a biological difference between physiological and pathological angiogenesis, TIE2 having a physiological role and TP being involved in pathological angiogenesis.

Key words: gene expression/pulsed Doppler spectral analysis/RT–PCR/thymidine phosphorylase/TIE2

Introduction

Transvaginal colour and pulsed Doppler ultrasonography has enabled studies of the dynamic vascular patterns in ovarian tumours (Bourne et al., 1989; Hata et al., 1992, 1995). Analyses of the difference between blood flow velocity waveforms has suggested that intra-tumoral peak systolic velocity (PSV) is the best predictor of ovarian malignancy (Hata et al., 1995; Tailor et al., 1996). Moreover, the PSV has been shown to be significantly associated with the development of healthy follicles (Collins et al., 1991; Campbell et al., 1993) and the endocrine function of the corpus luteum (CL) (Bourne et al., 1996). It is well known that transvaginal ultrasonography with colour flow imaging can be used to study intra-ovarian angiogenesis, which is responsible for follicular and CL formation (Campbell et al., 1993), and tumour development (Hata et al., 1997). Although PSV is a measure of recent vascular activity, we assume that it is directly related to the amount of vascular development, i.e. angiogenesis.

A growing tumour where the cells are stimulating new capillary sprouts and then accumulating around them appears to be analogous to a developing organ in the embryo. However, the analogy is imperfect because tumours continue to alter their intrinsic vasculature in a way that normal organs do not (Folkman, 1985). These angiogenic processes are probably induced by factors from within the tumour cell (Folkman et al., 1971). One such factor is platelet derived-endothelial cell growth factor (PD-ECGF), which was initially reported to be mitogenic for human umbilical vein endothelial cells, and subsequently shown to be thymidine phosphorylase (TP) (Furukawa et al., 1992; Moghaddam et al., 1995). It was reported that TP also has chemotactic and angiogenic activity (Haraguchi et al., 1994).

The analysis of mRNA for putative angiogenic factors in ovarian malignancies revealed the over-expression of TP/PD-ECGF compared with tissue from benign tumours and the normal ovary (Reynolds et al., 1994). Subsequently, the concentration of TP in ovarian tumour tissue was shown to be positively correlated with the PSV measured by pulsed-Doppler ultrasound (Hata et al., 1997). Although a relatively high PSV was recorded in CL, the amount of TP was low (Hata et al., 1997). In normal tissues, TP has various subcellular locations and a heterogeneous pattern of expression with no strong correlation to the expected sites of angiogenesis. TP might become angiogenic under particular pathological conditions, e.g. in tumours (Fox et al., 1995). Therefore, it is anticipated that another factor is responsible for angiogenesis in CL.

Physiological angiogenic processes in the adult are restricted to the female reproductive system where they occur cyclically during the ovarian and uterine cycle as well as during pregnancy (Reynolds et al., 1992). In contrast to pathological angiogenesis, blood vessels in physiological angiogenesis maintain their ability to grow and regress throughout life. Sprouting endothelial cells invade the...
growing CL and continue to grow throughout the first third of the ovarian cycle. Thereafter, the mature CL is characterized by a dense network of vessels with gradually decreasing vessel density (Augustin et al., 1995). An understanding of the difference in the angiogenic process between CL and ovarian cancer would not only increase our knowledge of pathological and physiological angiogenesis in the ovary but also might point the way to new forms of therapeutic intervention of ovarian cancer.

Tie2 (also known as Tek) is a novel endothelium-specific receptor tyrosine kinase, which has been demonstrated to be essential for development of the embryonic vasculature (Suri et al., 1996; Partanen and Dumont, 1999). It has been suggested that Tie2 may have a dual function in both vascular growth and vascular maintenance in adult tissues (Wong et al., 1997). Immunoprecipitation and RNase protection assay confirmed Tie2 expression in healing skin wounds, demonstrating rapid up-regulation of Tie2 protein and mRNA during the early stages of wound healing, coinciding with wound angiogenesis, and rapid down-regulation of Tie2 during the later stages, coinciding with regression of vessels (Wong et al., 1997). Ovarian follicle development depends on the sequential regulation of vascular outgrowth and vascular regression. At maturation, the follicle ruptures, expels the ovum, and then undergoes reorganization into a cell-dense secretory structure known as the CL. This process includes a wave of vascular sprouting and ingrowth that hypervascularizes the CL; these vessels eventually regress as the CL ages (Maisonpierre et al., 1997). These processes of growth and regression of CL show similarities to those of wound healing.

Therefore, we have examined mRNA expression of TP and TIE2 using reverse transcriptase–polymerase chain reaction (RT–PCR) in 14 normal ovaries with CL and nine with ovarian cancer in this study. The aim of this study was to compare the expression of these two genes in normal ovary with CL and in ovarian cancer, and to compare the results with ultrasound-derived PSV, as an indicator of angiogenesis (Campbell et al., 1993; Hata et al., 1997).

### Materials and methods

#### Experimental design

The plan was to recruit and scan patients within 24 h before laparotomy for a suspected gynaecological disorder. These women were electively admitted to our gynaecology ward. Asymptomatic women with a positive result from an ovarian cancer screening programme were excluded. If the ovaries were removed for diagnostic or therapeutic purposes, then a portion of tissue was taken for the method previously described (Hata et al., 1997, 1999).

Pelvic ultrasonography

Aloka SSD-2000 scanner with a transvaginal probe containing a 5.0 MHz transducer was used (Aloka Co, Tokyo, Japan). The direction of blood flow relative to the transducer, within a 22–80° convex, was indicated by shades of red or blue. The lowest measurable blood velocity in the colour or pulsed Doppler mode was 1.54 cm/s. The pulse repetition frequencies were 1–25 kHz; the maximal penetration depth was 12 cm, and the gate width ranged from 1 to 10 mm. The wall filter (50 Hz) was used to eliminate low frequency signals from movements of the vessel wall. The spatial peak temporal average intensity at the maximum amplitude and minimum gate width in simultaneous colour and pulsed Doppler mode was < 80 mW/cm² according to the manufacturer’s specifications. The scanning procedure has been described previously (Hata et al., 1992).

Colour Doppler imaging was used to identify areas of increased intra-ovarian blood flow which were recorded on an electronic colour print. In the case of CL, the colour map formed a continuous ring around CL. In the cancers, the pattern was patchy, and colour occurred predominantly in the septa and solid areas. An electronic gate was systematically placed over the area of interest and flow velocity waveforms were recorded. The highest PSV and the corresponding resistance index (RI) were determined. The average values from five consecutive reproducible waveforms were used for subsequent statistical analysis. The area with the lowest RI was chosen for tissue sampling after surgery. The position of the chosen area was recorded on diagrams to assist tissue collection. This method has been previously reported (Reynolds et al., 1994; Hata et al., 1997).

#### Tissue specimen and RNA preparation

Fresh surgical specimens from all patients were obtained, and the tissues for investigation were prepared carefully under a microscope to dissect ovaries into cancerous tissue, and healthy tissue around the CL and areas of high blood flow velocity. The tissue samples were stored at −80°C for subsequent analysis. As positive control for TP and TIE2 gene expression, normal liver and placental tissues were used respectively and the breast carcinoma cell line, MCF-7, which was kindly provided by Dr Akira Yamauchi, was used as negative control for both types of gene expression.

#### RT–PCR

TIE2 and TP expression were determined by RT–PCR, according to the method previously described (Arão et al., 1994). Briefly, complementary DNA (cDNA) was prepared by random priming from 500 ng of total RNA using a First-Strand cDNA Synthesis kit (Pharmacia-LKB, Uppsala, Sweden). The primers for the human TP gene (GenBank accession number M63193) amplification was TGGCTCAGTCGGGACAGCAG (upstream) and TCCGCTGATCATTGCCACCT (downstream) (152 bp of PCR product) (Hata et al., 1999); and for the human TIE2 gene (GenBank accession number L06139) AACTCTGTGTGCAACTGGTCC (upstream) and AAGT-
CATCTCCGAGCTTGG (downstream) (181 bp). The PCR was carried out in a Thermal Cycler (Perkin-Elmer Cetus, Northwalk, CT, USA) with a mixture consisting of complementary DNA (cDNA) derived from 5 ng of RNA, 10 pmol of upstream and downstream primers for each gene and 5 pmol of primers for the β2-microglobulin (β2-MG) gene (GenBank accession number, U00567; upstream primer: ACCCCCACTGAAAAAGATGAG; downstream primer: ATCTTCAAACCTCCATGATGC producing a 120 bp fragment), 200 µmol of deoxynucleotide triphosphate, 37 kBq of [α-32P]-dCTP, and 0.1 IU of Taq DNA polymerase with reaction buffer (Life Technologies, Rockville, MD, USA) in a final volume of 10 µl. The PCR conditions were denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min. A total of 30 cycles of PCR were performed for each specimen, and the products were separated on 9% polyacrylamide gels. Then radioactivity was determined using a BAS 2000 Bioimage Analyzer (Fujix, Tokyo, Japan). TP and TIE2 expression were presented as the relative yield of each gene to that of β2-HG.

**Statistical analysis**

The Mann–Whitney U-test was used to evaluate significant differences between endpoints, while regression analysis was used to determine the correlation between end-points. P < 0.05 was considered to be statistically significant.

**Results**

**RT–PCR and TP and TIE2 gene expression**

To determine the number of PCR cycles appropriate for quantification, PCR was performed from 20 to 50 cycles in increments of five cycles. The expression ratios of TP and TIE2 to β2-HG were reasonably constant from 25 to 40 cycles respectively (data not shown). Therefore, in subsequent experiments, the values at 30 PCR cycles were defined as the expression of target genes. The representative profile of each gene expression by RT–PCR is shown in Figure 1.

**PSV and TP and TIE2 gene expression**

The relative gene expression for TP and TIE2 are the mean values from at least three independent RT–PCR experiments. There was no significant difference between PSV in normal ovary with CL and that in ovarian cancer (median 28.6, range 12.8–42.3 cm/s versus median 26.8, range 13.8–49.3 cm/s respectively; P = 0.95, Mann–Whitney U-test) (Figure 4). The value of TP gene expression in ovarian cancer was significantly higher than that in normal ovary with CL (median 1.14, range 0.28–2.80 versus median 0.38, range 0.12–1.70 respectively; P = 0.02, Mann–Whitney U-test) (Figure 3). No significant difference was noted between the value of TIE2 gene expression in the normal ovary with CL (median 0.20, range 0.10–0.47) and that in ovarian cancer (median 0.15, range 0.04–0.35) (P = 0.186, Mann–Whitney U-test) (Figure 4). There was a significant correlation between the TIE2 gene expression and the PSV in normal ovary with CL (r = 0.633, P = 0.015) (Figure 5), but the corresponding relationship between TP gene expression and the PSV was not signi-
Figure 4. Relative Tie2 gene expression in normal ovary with corpus luteum (CL) and in ovarian cancer. Horizontal lines indicate median values.

Figure 5. Relationship between Tie2 gene expression and peak systolic velocity in normal ovary with corpus luteum.

Figure 6. Relationship between thymidine phosphorylase (TP) gene expression and peak systolic velocity in ovarian cancer.

The present results confirm our previous findings (Hata et al., 1997), that intratumoral PSV reflects production of TP in ovarian cancer, and is indicative of angiogenesis. Although the difference was not found to be significant, the value of TIE2 gene expression in CL was higher compared with that in ovarian cancer. There was a significant correlation between TIE2 gene expression and PSV in CL, but the corresponding relationship between TP gene expression and PSV was not found to be significant. Of course, the expression of TIE2 and TP may vary depending upon the stage of corpus luteum development. Unfortunately, the precise time in the menstrual cycle of the patients was not recorded. Nevertheless, transvaginal colour Doppler spectral analysis of the peri-ovulatory phase revealed that the PSV monitors intra-ovarian angiogenesis (Campbell et al., 1993). Therefore, the TIE2 gene might be one of the key genes that modulate angiogenesis in CL formation and regression, e.g. in physiological angiogenesis.

Angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) are novel 75 kDa secreted ligands for the vascular endothelial-restricted receptor tyrosine kinase, Tie2 (Maisonpierre et al., 1997). Although they bind with comparable affinity, Ang1 induces

**Discussion**

In this study, no significant difference was found between PSV in CL and that in ovarian cancer. This means that at this point in the menstrual cycle, the Doppler study cannot discriminate between physiological and pathological angiogenesis. This appears to contradict the finding of previous studies (Hata et al., 1995; Tailor et al., 1996). However, the results may depend upon the time of measurement. In order to determine ovarian malignancy, PSV should be measured on days 5–7 of the menstrual cycle in order to avoid the effect of ovulation and the presence of the CL. The value of TP gene expression in ovarian cancer was significantly higher than that of TP gene expression in CL. TP gene expression was correlated significantly with the PSV in ovarian cancer but not with that in CL. Polymerase chain reaction (PCR), is a highly sensitive and efficient method of amplifying specific DNA segments present at low concentrations, and provides an alternative approach for estimating the relative gene expression in small amounts of tissues (Eisenstein, 1990). By amplifying cDNA reverse-transcribed from RNA, PCR can be used to measure quantitatively the expression of specific genes in tumour cells (Arao et al., 1994). While gene expression is not a direct measure of enzyme activity, it have been shown that when gene amplification takes place, it is closely related to increases in enzyme concentrations, gene copy number, and mRNA values (Curt et al., 1983). Moreover, a significant correlation between TP enzyme activity and TP mRNA expression measured by RT–PCR was reported (Mimori et al., 1997).
Tie2 phosphorylation, whereas the cognate ligand Ang2 can competitively block the effects of Ang1. Ang2 dose-dependently blocks directed migration of endothelial cells toward Ang1 in vivo, consistent with the role of Ang2 as a naturally occurring inhibitor of Ang1 (Witzenbichler et al., 1998). This suggests that they are agonists and antagonists of the Tie2 signalling cascade in the vascular endothelium. RT–PCR analysis of Ang1 and Ang2 expression, using tissue specimens of bovine ovaries, revealed that both molecules are expressed throughout the ovarian cycle. The quantitative Ang2/Ang1 ratio does, however, change from 1.34 in angiogenic CL and 1.07 in mid-stage CL to 7.59 during CL regression, reflecting the strong overexpression of Ang2 over Ang1 during blood vessel regression (Goede et al., 1998). These findings are compatible with the finding that the correlation between Ang2 expression and blood vessel regression was demonstrated in human adult ovarian tissue (Maisonpierre et al., 1997). Although we did not evaluate the mRNA expression of Ang1 and Ang2 in human CL in this study, the significant correlation between the mRNA expression of Tie2 and the PSV might reflect the relationship between the complexity of Ang1 (receptor activator) and Ang2 (receptor antagonist)/Tie2 (receptor) signal and PSV in CL.

We have suggested that inhibitors of TP suppress the growth of the tumours by inhibiting the angiogenic activity of the enzyme and the inhibition of TP offers a new modality for the treatment of ovarian cancer (Hata et al., 1997). If we anticipate the transfection of Ang1 and Ang2/TIE2 gene levels similar to those seen during CL regression, in addition to anti-TP therapy in ovarian cancer, then the measurement of PSV by pulsed Doppler spectral analysis might be a useful endpoint for assessing the magnitude of the response. Recently an important interaction between vascular endothelial growth factor (VEGF), and Ang1 and Ang2 has been suggested (Asahara et al., 1998; Holash et al., 1999). VEGF also acts via members of a family of endothelial-specific receptor tyrosine kinases (Suri et al., 1996). The study of the Ang1 and Ang2/TIE2 gene expression in concert with VEGF gene expression during the ovarian cycle warrants further exploration.

References


